JOURNAL OF VIROLOGY, Feb. 2007, p. 1230–1240 Vol. 81, No. 3
0022-538X/07/$08.00+0 doi:10.1128/JVI.01586-06
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

The ERK Mitogen-Activated Protein Kinase Pathway Contributes to Ebola Virus Glycoprotein-Induced Cytotoxicity▽

Carisa A. Zampieri,[1] Jean-Francois Fortin,[2]† Garry P. Nolan,[2] and Gary J. Nabel[1]*

Vaccine Research Center, NIAID, National Institutes of Health, Room 4502, Bldg. 40, MSC-3005, 40 Convent Drive, Bethesda, Maryland 20892-3005,[1] and Baxter Laboratory of Genetic Pharmacology, Dept. of Microbiology and Immunology, Stanford University School of Medicine, 269 Campus Drive, CCSR, Rm. 3205, Stanford, California 94305-5175[2]

Received 24 July 2006/Accepted 6 November 2006

Ebola virus is a highly lethal pathogen that causes hemorrhagic fever in humans and nonhuman primates. Among the seven known viral gene products, the envelope glycoprotein (GP) alone induces cell rounding and detachment that ultimately leads to cell death. Cellular cytoxicity is not seen with comparable levels of expression of a mutant form of GP lacking a mucin-like domain (GPΔmuc). GP-induced cell death is nonapoptotic and is preceded by downmodulation of cell surface molecules involved in signaling pathways, including certain integrins and epidermal growth factor receptor. To investigate the mechanism of GP-induced cellular toxicity, we analyzed the activation of several signal transduction pathways involved in cell growth and survival. The active form of extracellular signal-regulated kinases types 1 and 2 (ERK1/2), phospho-ERK1/2, was reduced in cells expressing GP compared to those expressing GPΔmuc as determined by flow cytometry, in contrast to the case for several other signaling proteins. Subsequent analysis of the activation states and kinase activities of related kinases revealed a more pronounced effect on the ERK2 kinase isoform. Disruption of ERK2 activity by a dominant negative ERK or by small interfering RNA-mediated ERK2 knockdown potentiated the decrease in αV integrin expression associated with toxicity. Conversely, activation of the pathway through the expression of a constitutively active form of ERK2 significantly protected against this effect. These results indicate that the ERK signaling cascade mediates GP-mediated cytotoxicity and plays a role in pathogenicity induced by this gene product.

Ebola virus is an enveloped, negative-strand RNA virus in the family *Filoviridae*, which is capable of inducing severe hemorrhagic fever syndrome in humans, nonhuman primates, and other species (37). Four species of the virus have been identified to date: Zaire, Sudan, Ivory Coast, and Reston. Of these, the highly pathogenic Zaire species produces mortality rates of up to 90% (23). Though the pathogenic determinants of Ebola virus remain incompletely defined, several lines of evidence suggest that the viral glycoprotein (GP) is a key contributor to the adverse events of infection. Expression of Ebola virus GP from all four subtypes induces variable degrees of cytotoxicity in cell lines and primary cells in vitro that is characterized by cell rounding and detachment, followed by cell death (47). Although there is some debate over the role of GP cytotoxicity during live viral infection (2, 18, 35), differences in GP-induced cytotoxicity are reflected in the mortality rates caused by the different subtypes (40, 47), indicating the importance of this gene product in the pathogenic course of the disease.

Membrane-associated Ebola virus GP is a heavily glycosylated type I transmembrane protein that is responsible for receptor binding and fusion of the virus with host cells (9, 37, 46). During assembly, a GP precursor (GP0) is cleaved by a furin-like protease into GP1 and GP2 subunits to form a heterodimer. Trimers of the GP1/GP2 heterodimer form the sole structural protein on the surface of the virion (44). Transient expression of Ebola virus GP induces loss of adherence from the extracellular matrix in several different cell lines and primary cell types, including cell types infected by Ebola virus in vivo (8, 40–42, 46). The morphological changes observed upon GP expression in vitro correlate with the presence of a mucin-like domain (46). Expression of wild-type GP, but not comparable levels of GP lacking the mucin-like domain (GPΔmuc), decreases the presence of cell surface molecules important for cell adhesion, signaling, and immune evasion. These transmembrane glycoproteins include certain integrin subunits, epidermal growth factor receptor (EGFR), and major histocompatibility complex class I (40–42). Since integrin-mediated adhesion is important in cell-matrix interactions and intracellular signaling, it is thought that the decreased surface expression of these important molecules may play a major role in the induction of rounding and detachment by GP (40, 41).

In this study, we examined the effects of Ebola virus GP expression on pivotal proteins in several cellular signal transduction pathways and investigated whether they contribute to GP-induced cytotoxicity. Using flow cytometric analysis of phosphoprotein expression, we surveyed the activation of several proteins important in cell growth and survival, including the mitogen-responsive extracellular regulated kinases 1 and 2 (ERK1/2) and p38, the stress-activated c-Jun-NH(2) terminal kinase (JNK), and the tumor suppressor protein p53. We found that GP expression was associated with a mucin domain-dependent reduction in active levels of mitogen-activated protein kinase (MAPK) effector ERK2, a kinase that is important

* Corresponding author. Mailing address: Vaccine Research Center, NIAID, National Institutes of Health, Room 4502, Bldg. 40, MSC-3005, 40 Convent Drive, Bethesda, MD 20892-3005. Phone: (301) 496-1852. Fax: (301) 480-0274. E-mail: gnabel@nih.gov.

† Present address: Research and Development, Boehringer Ingelheim (Canada) Ltd., 2100, Cunard St., Laval, Québec, Canada H7S 2G5.

▽ Published ahead of print on 15 November 2006.

in mediating cellular responses such as proliferation, cell cycle progression, and survival. Further analysis indicated a role for this kinase in GP-mediated cell rounding and detachment. Together these data provide insight into the signaling pathways modulated by Ebola virus GP and suggest a new potential therapeutic target against GP-mediated cytopathicity.

(This work is included in a dissertation presented to the Genetics Program of the Institute for Biomedical Sciences at the George Washington University, Washington, DC, by Carisa Zampieri in partial fulfillment of the requirements for the Ph.D. degree).

MATERIALS AND METHODS

Plasmids and cell culture. Expression vectors p1012, pGP(Z), and pGPΔmuc contain a cytomegalovirus enhancer promoter as previously described (46). The ERK1DN, ERK2-MEK1, ERK2-MEK1LA, and KRERK2-MEK1LA expression vectors were generously provided by Melanie H. Cobb (University of Texas Southwestern Medical Center, Dallas, TX). Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (GIBCO). Cells were transfected using either the Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) transfection reagent according to the protocol supplied by the manufacturer.

Flow cytometric detection of intracellular phosphoproteins. Cells (1.2×10^6) were seeded in a 10-cm^2 plate 1 day prior to transfection. The cells were transfected with 5 μg of plasmid by using Fugene 6 transfection reagent. At 12, 18, 24, and 36 h posttransfection, cells were harvested by pipetting up and down six times to resuspend both adherent and nonadherent cells. They were centrifuged for 5 min at $900 \times g$ and fixed by resuspension in cold phosphate-buffered saline (PBS) containing 2% paraformaldehyde to have a concentration of no more than 1×10^6 cells/ml. Cells were incubated at room temperature for 10 min, centrifuged for 5 min at $900 \times g$, and resuspended in cold PBS at a concentration of 10×10^6 cells/ml. Nine volumes of cold methanol were slowly added to the cell suspension during vortexing to permeabilize the cells. This fixation decreased the reactivity of antibodies with GPΔmuc relative to GP when equivalent levels were detected by cell surface staining. The cells were then incubated on ice for 30 min and stored at −80°C. To rehydrate the cells prior to staining, cells in methanol were diluted with 2 volumes of staining buffer, spun at $800 \times g$ for 10 min at 4°C, and then resuspended in staining buffer at a concentration of 10^7 per ml. Cells were stained for 30 min on ice with the indicated antibodies (see below) and washed once with staining buffer. Cells were analyzed on a FACSCalibur apparatus. Cells were stained with antibodies to GP (biotin-conjugated mouse monoclonal antibody followed by streptavidin-phycoerythrin) and phosphorylated proteins phospho-ERK1/2 (clone 20a, pT202/pY204), phospho-p38 MAPK (clone 36, pT180/pY182), phospho-p53 (polyclonal, pS46), and phospho-JNK1/2 (polyclonal, pT183/Y185) conjugated to either Alexa Fluor 647 or Alexa Fluor 488 (BD Biosciences).

Phospho-MAPK antibody arrays. 293 cells (1.2×10^6) were transfected in 10-cm plates with 5 μg of control vector, GP, or GPΔmuc plasmid by using Fugene 6 transfection reagent. An analysis of the phosphorylation states of all MAPKs was performed using a human phospho-MAPK array kit, equivalent to immunoprecipitation and Western blot analysis (R&D Systems, Minneapolis, MN). At 24 h after transfection, cells were rinsed with PBS and lysed with the buffer provided. Arrays were incubated overnight at 4°C with 250 μg of lysate from control-, GP-, or GPΔmuc-transfected cells. The arrays were washed three times with 20 ml of wash buffer provided and incubated for 2 h with the provided detection antibody cocktail containing phospho-site-specific MAPK biotinylated antibodies. The wash steps were repeated, after which the arrays were exposed to chemiluminescent reagents and film. The data on the developed X-ray film were scanned and quantitated using image analysis software (Quantity One).

ERK1 and ERK2 kinase assays. p44/42 kinase assays were performed using nonradioactive kits from Cell Signaling Technology (Beverly, MA). Briefly, 293 cells were transfected with control, GP, or GPΔmuc expression vectors. The cells were harvested and lysed with the provided 1× lysis buffer. The protein content in the lysates was determined by the Bradford method (7). Either 25 μg, 100 μg, 500 μg, or 1 mg of cell lysates was immunoprecipitated with polyclonal antibodies against total ERK1 (Upstate Cell Signaling Solutions) or ERK2 (Upstate Cell Signaling Solutions) by use of immobilized protein G (Invitrogen). The precipitated enzymes were then used for kinase assays with Elk-1 substrate followed by Western blot analysis with antibodies that allow detection and quantitation of phosphorylated substrate.

siRNA-mediated knockdown. The ERK2-specific short interfering RNAs (siRNAs) (siRNA-A [GGGUUCCUGACAGAAUAUGtt] and siRNA-B [GGAAAAGCUCAAAGAACUAtt]) and the negative control siRNA (control siRNA, *Silencer* negative control 1) were obtained from Ambion (Austin, TX). Lowercase letters indicate sequences not complementary to ERK2 but necessary for SiRNA duplex function. 293 cells (1×10^5) were transfected with 25 μM of each siRNA by using Lipofectamine transfection reagent. After a 24-h incubation, cells were transfected in the same manner with siRNA as well as 250 ng of plasmids expressing the control or GP. At 2, 3, and 4 days after the initial siRNA transfection, cells were harvested and analyzed for both GP cytotoxicity and ERK2-specific knockdown by using flow cytometry and Western blot analysis followed by quantitation (Quantity One), respectively, as described below.

Western blot analysis. Cell lysis for Western blotting was performed in cell lysis buffer (Cell Signaling Technology). Antibodies against GP (rabbit necropsy serum), ERK1/2 (Cell Signaling Technology), ERK2 (Cell Signaling Technology), and β-actin (Sigma) were used in immunoblotting in accordance with the manufacturer's instructions.

GP cytotoxicity assay. To quantitate GP-induced cytotoxicity, we measured the amount of αV integrin downregulation in GP-expressing cells as previously described (41). Briefly, 293 cells were transfected with the indicated plasmid vectors and analyzed by fluorescence-activated cell sorting (FACS) for cell surface expression of both Ebola virus GP and αV integrin surface molecules at 24 and 48 h after transfection. Both adherent and detached cells were collected and incubated with antibodies to Ebola virus GP and integrin αV for 30 min on ice. The cells were washed with 1 ml ice-cold PBS containing 2% fetal bovine serum and incubated with allophycocyanin (Jackson ImmunoResearch Laboratories)- or phycoerythrin (Sigma)-conjugated secondary antibodies for 30 min on ice. Cells were washed as described above and resuspended in PBS containing 1% paraformaldehyde. Analysis was conducted using a Becton Dickinson four-color Calibur flow cytometer and FlowJo analysis software.

RESULTS

Flow cytometric analysis of kinase phosphorylation in cells expressing Ebola virus GP. Ebola virus GP modulates the surface expression of cellular proteins, downregulating cell surface integrins, major histocompatibility complex class I, and other cell surface molecules (40–42). Since these proteins have been implicated in cellular signaling pathways, we analyzed the activation of several kinases and molecules involved in cell growth and survival to determine if perturbation in one or more signaling pathways correlated with GP cytotoxicity. Phosphoprotein flow cytometry was used to analyze the phosphorylation states of the signaling molecules ERK1/2, JNK, p38, and p53 in cells expressing Ebola virus GP. Human embryonic kidney 293 cells were transfected with plasmids expressing full-length GP, GPΔmuc, or a no-insert vector control, and phosphorylation states were analyzed at 12, 18, 24, and 36 h after transfection.

At 18 h after transfection, the expression of Ebola virus GP reduced the levels of phosphorylated ERK1/2 and, to a lesser extent, p38 but not those of JNK or p53 (Fig. 1A). At this time point, the ratio of high- to low-phosphorylated ERK1/2 among the GP-expressing cells was approximately 1 to 3 (Fig. 1A, top middle panel [15.3 versus 43.9%, right upper versus lower quadrant]). In contrast, this ratio was slightly greater than 1 in GPΔmuc-transfected cells (Fig. 1A, top right panel [23.6 versus 18.9%, right upper versus lower quadrant]). This effect was also apparent at the earliest, 12-h time point, when the ratio of high- versus low-phosphorylated ERK1/2 in GP-transfected cells was approximately 1 to 2 (Fig. 1B, middle panel [14.3 to 25.2%, right upper versus lower quadrant]). The reduction in ERK1/2 phosphorylation remained constant and did not change significantly beyond the 18-h time point when exam-

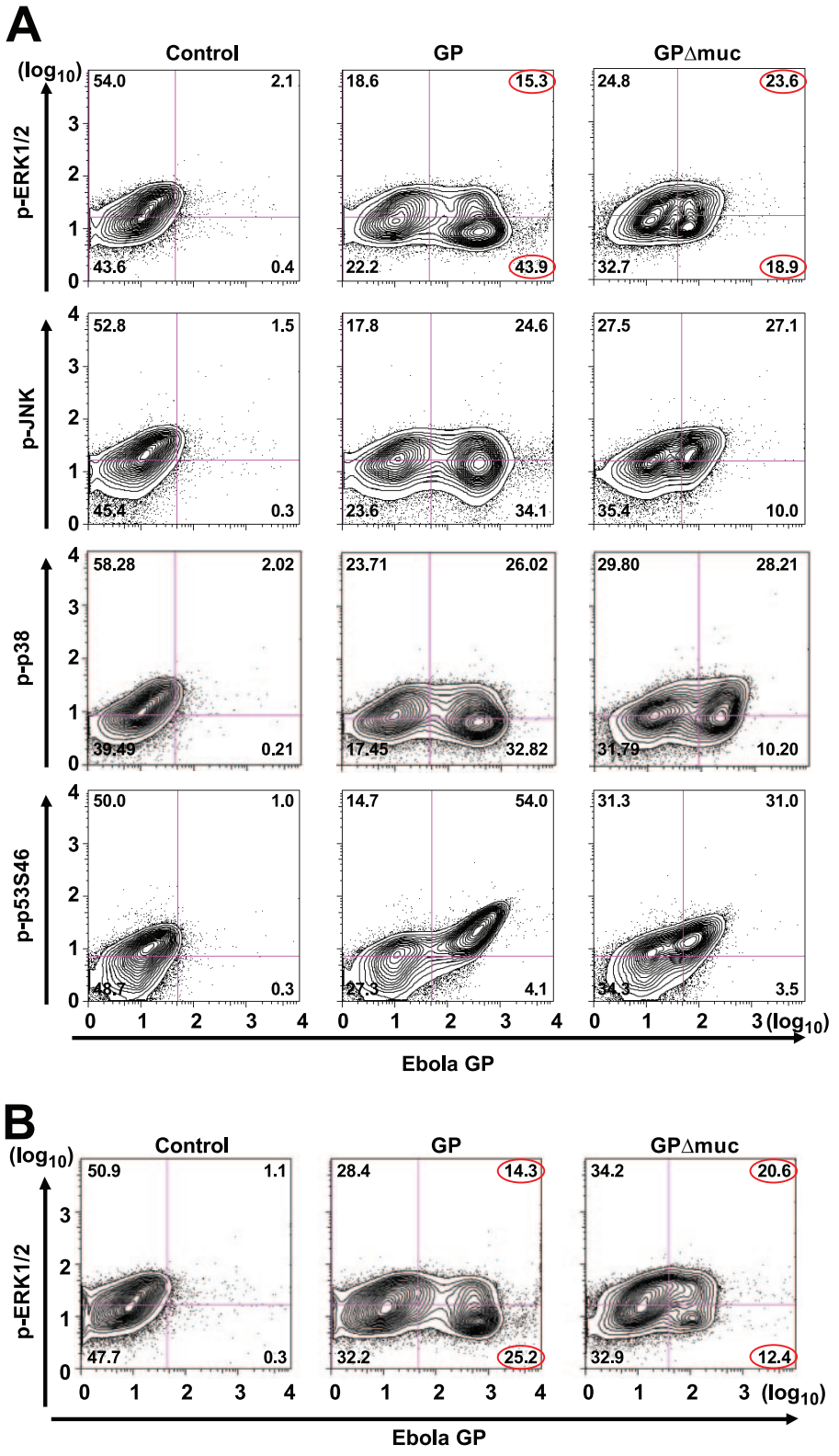

FIG. 1. Expression of Ebola virus GP decreases phospho-ERK1/2, the active form of this kinase. (A) 293 cells were transfected with a plasmid encoding a vector control, Ebola virus GP, or GPΔmuc. Both adherent and nonadherent cells were collected at 18 h after transfection. The cells were fixed, permeabilized, and stained with antibodies to Ebola virus GP and the phosphorylated forms of the signaling kinases ERK1/2, JNK, p38, and p53. (B) 293 cells were transfected and stained as described for panel A. Cells stained with antibodies against Ebola virus GP and phospho-ERK1/2 are shown at 12 h.

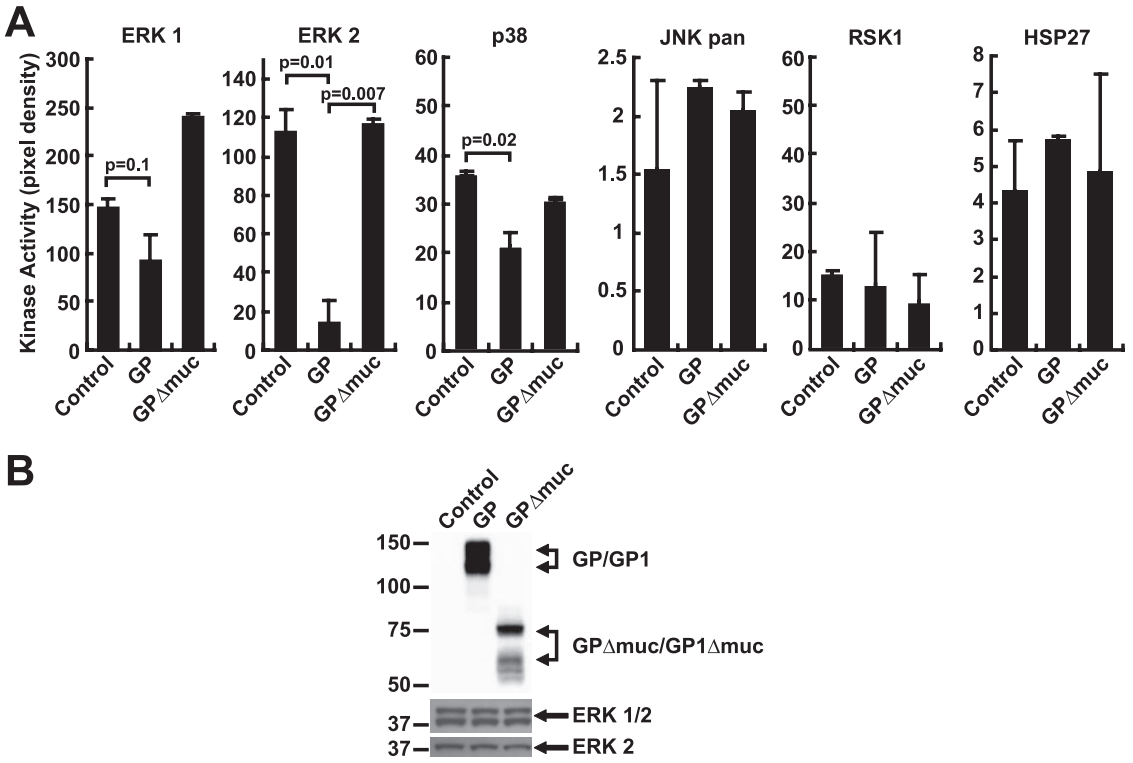

FIG. 2. Ebola virus GP specifically decreases the phosphorylation of the mitogen-activated protein kinase ERK2. (A) 293 cells were transfected with a plasmid encoding a vector control, Ebola virus GP, or Ebola virus GPΔmuc. After 24 h, both adherent and nonadherent cells were harvested, lysed, and analyzed for MAPK activity as described in Materials and Methods. The results are representative of two separate experiments done in duplicate. Error bars indicate standard deviations. (B) The cell lysates obtained from the experiment described for panel A were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were immunoblotted using antibodies specific for Ebola virus GP, total ERK1/2, and total ERK2. The two bands in the GP immunoblot correspond to the cleaved and uncleaved versions of the GP and GPΔmuc proteins as indicated.

ined at 24 and 36 h posttransfection (data not shown). An increase in the phosphorylation of the tumor suppressor gene p53 at residue S46 was also observed (Fig. 1A, lower row), but it occurred both in cells expressing GP and in those expressing GPΔmuc and therefore was deemed unlikely to be related to GP-induced cytotoxicity. GP expression had little effect on the activation state of the JNK kinase (Fig. 1A). Although a modest difference in the staining of intracellular GP and GPΔmuc was observed (Fig. 1A and B), this effect likely does not reflect real differences in the levels of these proteins in cells. The GP monoclonal antibody detects similar levels of GP and GPΔmuc cell surface staining (one- to twofold differences) in transfected cells. However, in order to measure intracellular kinase levels, the cells were fixed and permeabilized prior to staining. This procedure affected antibody binding to GPΔmuc more than that to GP (data not shown). Taken together, these results suggest that GP expression correlates with a specific reduction in the phosphorylation of ERK1/2 and that the perturbation of this kinase requires the mucin-like domain of GP.

A survey of the MAPK family reveals reduced ERK2 phosphorylation in cells expressing GP. ERK1 and -2 are members of the mitogen-activated protein kinase family. To determine whether GP expression modulated phosphorylation of other kinases in this family, we examined the activities of several related proteins. At 24 h after transfection, the levels of phosphorylated MAPK proteins in the cell lysates were analyzed using a human phospho-MAPK array. GP expression significantly reduced the amount of phosphorylated ERK2 compared to that with a vector control (Fig. 2A) ($P = 0.01$) and reduced ERK1 ($P = 0.1$) and p38 ($P = 0.02$) expression to lesser extents that were either not statistically significant (ERK1) or less substantial (p38). The effect on ERK2 was dependent on the mucin domain: cells expressing GPΔmuc had significantly higher levels of phosphorylated ERK2 than cells expressing full-length GP ($P = 0.007$). GP had little effect on other kinases, including JNK (Fig. 2A) and Akt and GSK-3β (data not shown). However, there were also no significant changes in the activation state of Rsk-1, a downstream substrate of ERK1 and ERK2. This may be due to the relatively low levels of Rsk-1 in unstimulated 293 cells, making changes in phosphorylation difficult to observe. In addition, GP expression did not alter phosphorylation of the unrelated heat shock protein 27 (Fig. 2A). Expression levels of the transfected plasmids and total ERK1 and ERK2 protein levels determined by Western blotting were the same in all lysates (Fig. 2B), indicating that the reduction in ERK2 phosphorylation was not the result of reduced kinase levels in the cells. The decreased intensity of GPΔmuc relative to GP on the Western blot is likely due to the presence of fewer antigenic determinants on the protein, because a significant portion is removed by the deletion mutation. These data demonstrate that GP exerts a more pro-

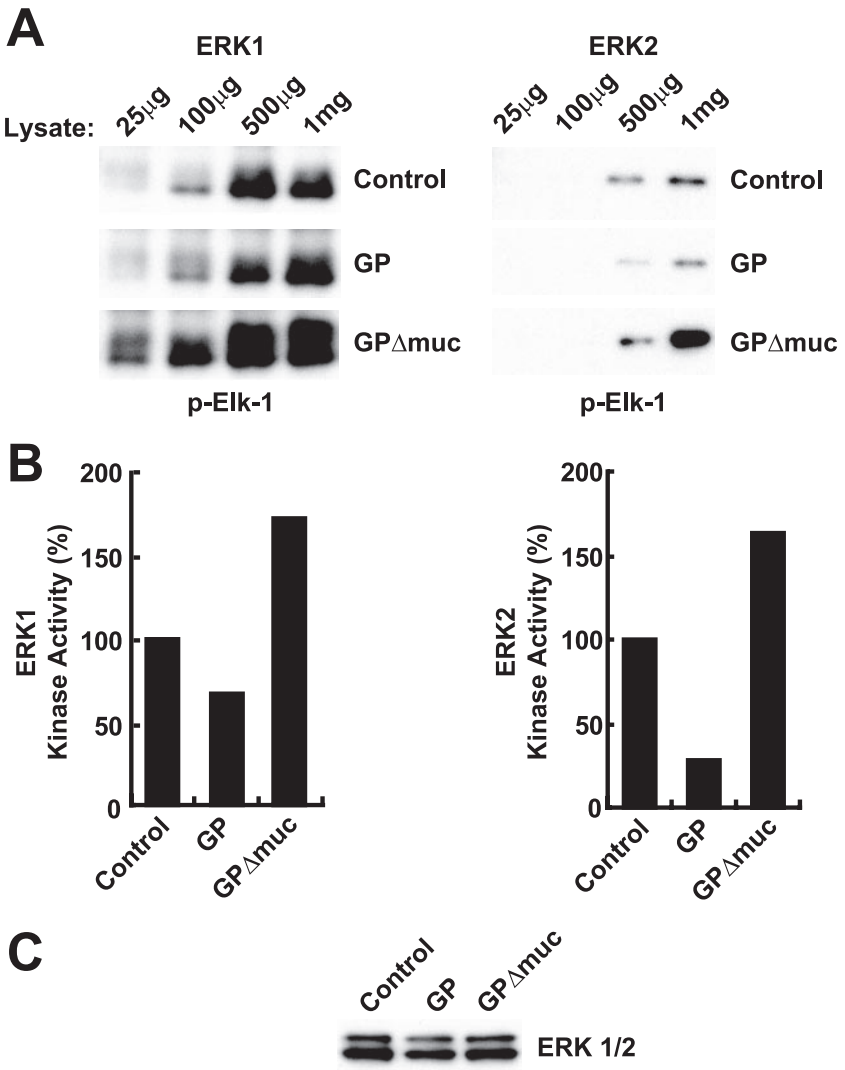

FIG. 3. Effect of GP expression on the kinase activities of ERKs. (A) 293 cells were transfected with a control plasmid lacking an insert, GP, or GPΔmuc expression vectors. Cells were harvested and lysed at 24 h after transfection. Total ERK1 and ERK2 were immunoprecipitated from increasing amounts of cell lysates, and the effects of GP expression on ERK1 and ERK2 kinase activities were determined as measured by phosphorylation of the substrate Elk-1. (B) The band intensities (500 μg lysate input) were quantitated to obtain percent kinase activities. (C) The cell lysates obtained from the experiment described for panel A were analyzed by Western blotting using an antibody specific for total ERK1/2.

nounced effect on the activation state of a specific MAPK, ERK2, which is mediated by the mucin-like domain.

ERK activity is inhibited by GP expression. ERK1 and ERK2 are closely related enzymes. In order to confirm a specific effect of GP on the activity of ERK2, we performed kinase assays with both ERK1 and ERK2 from control-, GP-, or GPΔmuc-transfected cells. Cells were harvested at 24 h after transfection, and various amounts of lysates were immunoprecipitated using antibodies against either ERK1 or ERK2. The immunoprecipitated kinases were incubated with the ERK1/2 substrate Elk-1. Elk-1 phosphorylation was then detected by Western blotting using an anti-phospho-Elk-1 antibody. Expression of GP reduced Elk-1 phosphorylation of both ERK1 and ERK2, but the reduction in ERK2 activity was more substantial and significant (Fig. 3A). Quantitation of band intensities from 500 μg of lysate revealed a 34% reduction in ERK1 activity versus a 72% reduction in ERK2 activity compared to control-transfected cells (Fig. 3B). The expression of GPΔmuc stimulated both ERK1 and ERK2 activities. This phenomenon was also observed in the MAPK array analysis of ERK1 phosphorylation (Fig. 2A). Taking these results together, we suggest that ERK1 may play a contributory role in GP toxicity, though it is likely to be necessary and not sufficient for this effect. Western blotting of cell lysates indicated that the alterations in kinase activities were not due to differences in the amounts of ERK1 and ERK2 proteins within the cells (Fig. 3C). These results suggest that GP expression decreases ERK2 and, to a lesser extent, ERK1 kinase activities.

Expression of a dominant negative ERK potentiates GP cytotoxicity. To investigate further the role of ERK activation on GP-induced cytotoxicity, the effects of a dominant negative ERK1 K71R (ERK1 DN) mutant protein were examined directly by counting the percentage of rounded and detached cells and also by flow cytometry to quantitate αV integrin

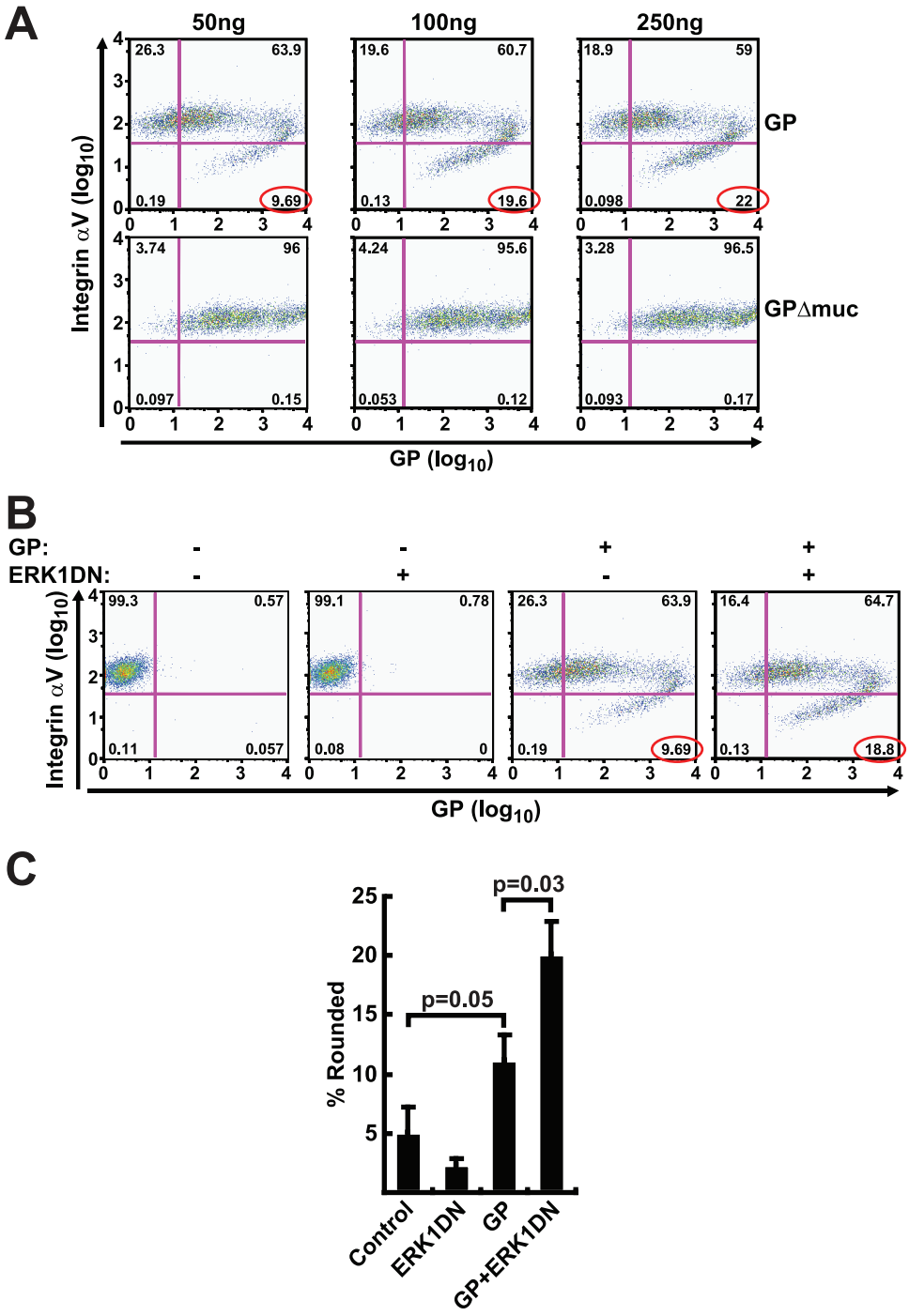

FIG. 4. A dominant negative form of ERK1/2 increases the cytotoxicity of Ebola virus GP. 293 cells were cotransfected with 50 ng, 100 ng, or 250 ng of Ebola virus GP or an empty vector control and 2 μg of ERK1 DN as indicated. Cells were incubated for 24 h, and both floating and adherent cells were counted to calculate percent rounding and then collected for staining with αV integrin and GP antibodies followed by FACS analysis. FACS results are shown for events in the live cell gate and are representative of at least three independent experiments. Error bars indicate standard deviations.

surface expression by comparison. The FACS-based assay was used to quantitate GP cytotoxicity because cell surface down-regulation of αV integrin has been shown to correlate with rounding and detachment in previous studies (40–42). Briefly, 293 cells were transfected with an empty vector control or a vector containing the dominant negative ERK1 DN. This mutant is deficient in kinase activity and binds to substrate, thus inhibiting the substrate's access to catalytically active ERK1 and ERK2 (25). Cells were cotransfected with increasing amounts of either an empty vector control or a GP or GPΔmuc expression vector. After 24 h, cells were harvested and dually stained for GP and αV integrin. Expression of GP decreased

surface αV integrin in a dose-dependent manner with a concomitant decrease in GP expression, giving a "comma" appearance (Fig. 4A, top row), possibly due to the surface removal of both αV integrin and GP, as noted previously (41). Deletion of the mucin-like domain, which eliminates toxicity, completely reversed the decrease in αV integrin levels (Fig. 4A, bottom row). In contrast, cells cotransfected with dominant negative ERK and GP showed a substantial increase in αV integrin downregulation. The number of cells with surface αV integrin increased substantially when cells were transfected with 50 ng of GP as well as the ERK1 DN construct (Fig. 4B, third panel versus fourth panel [9.69% versus 18.8%, lower right quadrant]). The expression of ERK1 DN alone had no effect on αV surface expression (Fig. 4B, second panel). To directly measure cytotoxicity, both adherent and rounding cells in Fig. 4B were counted prior to staining, and the percent rounding was calculated (Fig. 4C). At the lowest GP dose, GP expression increased the percentage of rounded cells, compared to less than 5% in control-transfected cells (Fig. 4C) ($P = 0.05$). The addition of ERK1 DN further increased cell rounding to approximately 20% (Fig. 4C) ($P = 0.03$). The percent rounding corresponded well to the quantitation of cytotoxicity by using the FACS-based approach and indicate that this indirect approach provides consistent and accurate measurements of GP-induced cytotoxicity. Together, these results suggest that inhibition of active ERK1/2 through the expression of a dominant negative ERK potentiates GP-induced toxicity as measured by both downregulation of αV integrin surface molecules and cell rounding.

siRNA-mediated knockdown of ERK2 increases the GP-induced phenotype. Since ERK1 and ERK2 sequences are highly homologous within their catalytic cores, the dominant negative ERK1 K71R protein can inhibit both protein kinase activities (10, 25). To determine whether the reduction in endogenous ERK2 function alone was sufficient to enhance GP-induced cell rounding and detachment, two ERK2-specific siRNA duplexes, ERK2 siRNA-A and ERK2 siRNA-B, directed against different regions of ERK2 mRNA were utilized to specifically decrease endogenous ERK2 protein expression. These two siRNA constructs were transfected in parallel with a negative control siRNA into 293 cells. After 24 h, cells were transfected again with the same siRNA duplexes and with plasmids expressing GP or a control empty vector plasmid. Cells were collected at 48, 72, and 96 h after the initial siRNA transfection, and at each time point the amount of GP-induced αV integrin downregulation was quantified by flow cytometry. The specificity of the ERK2 siRNAs was confirmed by Western blot analysis (Fig. 5A). At 96 h, ERK2 protein levels were reduced to 8% and 3% of normal levels (compared to control siRNA) with ERK2 siRNA-A and ERK2 siRNA-B, respectively. At this time, cells expressing GP and treated with the siRNAs demonstrated greater αV integrin downregulation than that induced by GP with control siRNA. Indeed, there was approximately threefold and twofold more downregulation of αV integrin in cells treated with ERK2 siRNA-A (Fig. 5B, top left versus top middle panel [8.25 versus 26%, right lower quadrant]) and ERK2 siRNA-B (Fig. 5B, top left versus top right panel [8.25 versus 19.9%, right lower quadrant]), respectively. ERK2 knockdown had minimal effects on αV integrin surface levels when transfected with vector control (Fig. 5B, lower panel). The results are representative of two independent experiments using three different amounts of GP. Taken together, these results suggest that the reduction in cellular ERK2 levels enhances the cytotoxicity induced by GP.

Expression of a constitutively active ERK2 reduces GP-induced cytotoxicity. Since an increase in GP-induced cytotoxicity was observed when ERK2 activity was diminished, we examined whether constitutive activation of ERK2 signaling would decrease this effect. Robinson et al. previously demonstrated that the fusion of wild-type ERK2 to its upstream regulator MAPK, MEK1 (ERK2-MEK1), produces a constitutively active form of ERK2 (32). A nuclear form of this fusion protein (ERK2-MEK1LA) is active in the absence of extracellular signals and does not activate endogenous ERK1 or ERK2. The expression of this fusion protein is sufficient to cause several transcriptional and phenotypic responses in mammalian cells. Expression of inactive ERK2 fused to the upstream regulator (KRERK2-MEK1LA) eliminates the catalytic activity of the fusion protein and acts as a negative control for the constitutively active fusion protein (32). We examined the effects of the expression of these constructs on GP cytotoxicity by cotransfecting 293 cells with expression vectors encoding GP and either ERK2-MEK1, ERK2-MEK1LA (active ERK2), or KRERK2-MEK1LA (inactive ERK2). Cells were incubated for 24 h, after which the amount of cytotoxicity was quantified with the flow cytometric assay for αV integrin downmodulation. None of the three fusion proteins, ERK2-MEK1, ERK2-MEK1LA, and KRERK2-MEK1LA, had an effect on basal αV integrin surface expression in the absence of GP (Fig. 6, upper row). Cells cotransfected with constitutively active ERK2 and GP showed 50% less surface downregulation of αV integrin than cells transfected with GP alone (Fig. 6, first versus third lower panel [23.2 versus 56.6%, right lower quadrant]). In contrast, expression of the kinase-inactive KRERK2-MEK1LA fusion protein had no effect on αV integrin downmodulation by GP (Fig. 6, lower row, first versus fourth panel [56.6 versus 52.9%, right lower quadrant]). In addition, cells cotransfected with GP and the cytoplasmic form of the fusion protein (ERK2-MEK1) had a more modest decrease in αV integrin surface expression than cells transfected with GP alone (Fig. 6, lower row, first versus second panel [56.6 versus 34.8%, right lower quadrant]). These data demonstrate that GP-mediated αV integrin downmodulation and cytotoxicity are diminished by expression of constitutively active ERK2.

DISCUSSION

The envelope protein of Ebola virus has been shown to mediate cell rounding and detachment in vitro and in vivo and has been implicated in the pathogenicity of Ebola virus infection (41, 47). Ebola virus GP cytotoxicity correlates with the downmodulation of several surface proteins important in adhesion and signaling, including certain integrins and EGFR (40–42). It is also dependent on recycling and endocytosis mediated by the cytoskeletal modulator dynamin (41). Although GP expression induces changes in cell surface protein levels, the mechanisms of surface protein downmodulation and cytotoxicity, as well as the cellular signaling events that mediate these effects, have not been defined. This study has analyzed the activation of several proteins that are pivotal to normal cell

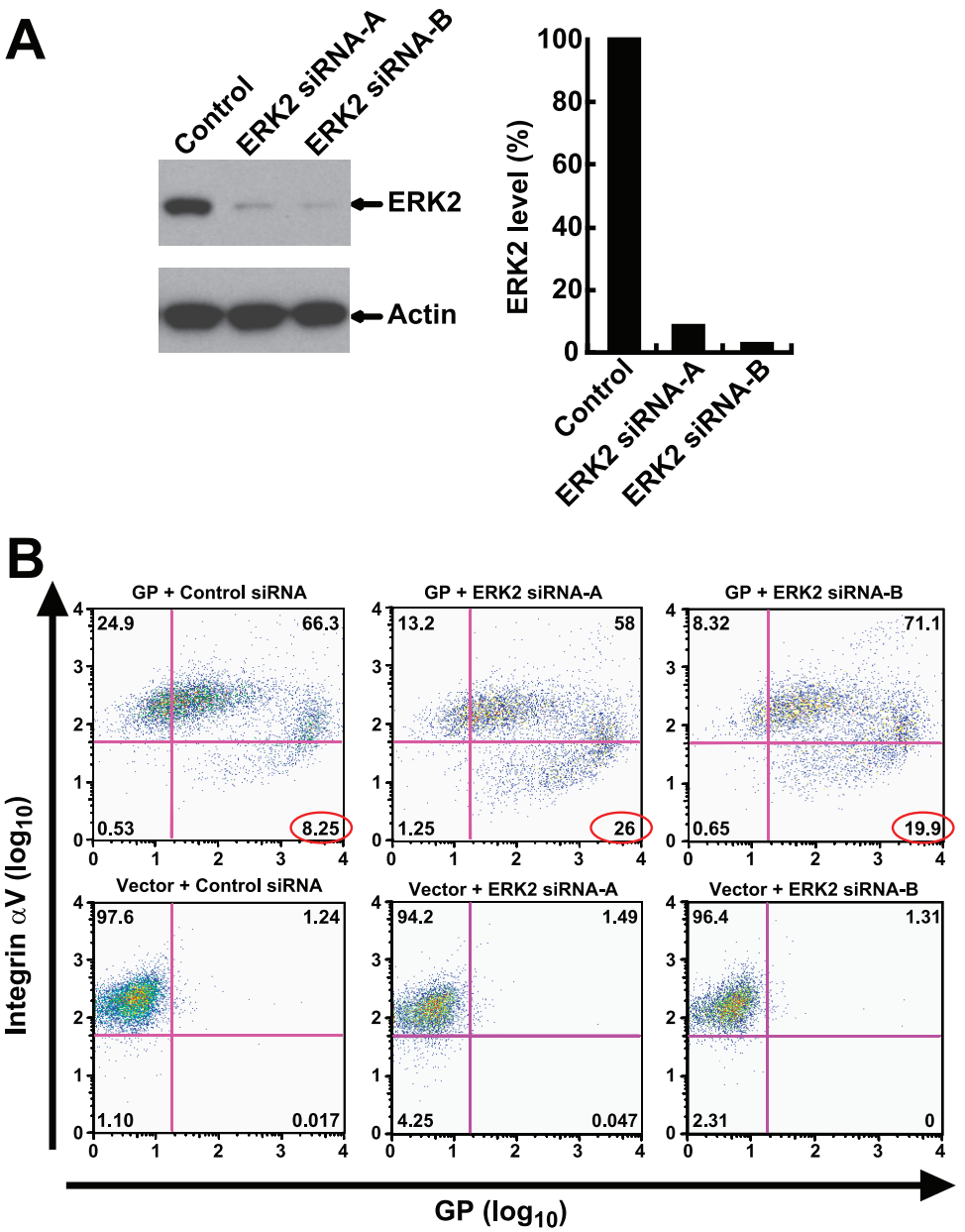

FIG. 5. Ebola virus GP cytotoxicity is increased by siRNA-mediated knockdown of ERK2. 293 cells were transfected with 25 μmol of control siRNA, ERK2 siRNA-A, or ERK2 siRNA-B, and 24 h later cells were transfected again with 250 ng of plasmid encoding vector control or Ebola virus GP and another 25 μmol of control siRNA, ERK2 siRNA-A, or ERK2 siRNA-B. The cells were incubated for another 72 h, after which both floating and adherent cells were collected and either lysed for Western blot analysis of ERK2 knockdown (A) or double stained with GP and αV integrin antibodies followed by flow cytometric analysis (B). Results are representative of two independent experiments.

signaling. Ebola virus GP significantly decreased the activation of ERK2, and this effect was dependent on the mucin-like domain of the glycoprotein, which is also required for cellular cytotoxicity (40, 42, 47). Inhibition of ERK2 activity, either through the overexpression of a catalytically inactive dominant negative form of ERK1/2 or through specific knockdown of endogenous ERK2, enhanced GP-induced αV integrin downregulation. Conversely, expression of a constitutively active ERK2 significantly decreased αV integrin downmodulation. Together, these data demonstrate that GP mediates its effect on cellular viability and integrin expression at least in part through ERK2 activity.

Cell adhesion is essential for proper cellular gene expression, growth, differentiation, and survival (19, 22, 38, 39). Integrins are pivotal to cell-cell and cell-matrix adhesion. Previously appreciated primarily for their role in the maintenance of adhesion, they are now well recognized as signaling receptors that regulate the cellular response to mitogens and intersect other signaling networks such as the ERK/MAPK cascade (22, 39). Several studies have shown that there is strong and sus-

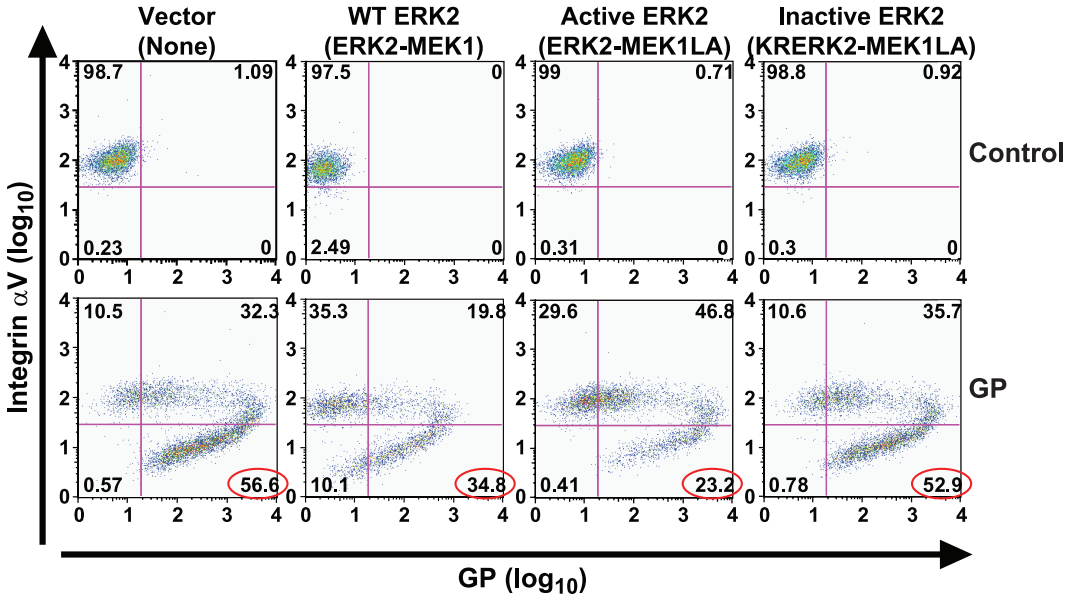

FIG. 6. Expression of a constitutively active ERK2 decreases Ebola virus GP-induced cytotoxicity. 293 cells were cotransfected with 250 ng of plasmid encoding a vector control (upper row) or Ebola virus GP (lower row) and 1 μg of either vector control, ERK2-MEK1, ERK2-MEK1LA, or KRERK2-MEK1LA as indicated and were incubated for 24 h. Supernatant cells were collected and pooled with adherent cells, double stained with antibodies specific for Ebola virus GP and αV integrin, and analyzed by flow cytometry. Results are shown for events in the live cell gate and are representative of at least three independent experiments. WT, wild type.

tained ERK activity in adherent cells (reviewed in references 4 and 5). In contrast, endothelial cells treated with integrin αVβ3 agonists in vivo show diminished long-term ERK activation (15). GP expression substantially reduced surface αV integrin levels as well as a loss of cell adherence. It may be that the observed GP-induced reduction in ERK activity is an upstream signaling event which subsequently leads to surface integrin downmodulation. However, given the importance of cell adhesion and integrins in the regulation of the ERK pathway, it is likely that the diminished ERK signaling is downstream of GP-induced cell rounding and detachment. This effect was most pronounced in ERK2 and was less obvious in other mitogen-activated kinases such as JNK and p38, which are not regulated by integrin-mediated adhesion (3).

Our results suggest that GP affects the catalytic activity of the ERK2 protein. Given the 80% sequence homology between ERK1 and ERK2 (10) and their similar modes of regulation, the preferential effect on ERK2 was not anticipated; however, several studies have shown more recently that despite their sequence homology, these two proteins have unique properties (16, 36, 43). For instance, although the proteins are coexpressed in virtually all tissues, their relative abundances vary widely. In addition, ERK1-deficient mice have a different, significantly milder phenotype than ERK2-deficient mice, which die early in development (20, 29). These recent findings suggest that ERK1 and ERK2 are not redundant proteins and that they have individual functions in cell signaling. In addition, the results presented here show a similar effect, to a lesser degree, on ERK1, Thus, the finding that GP expression has a more pronounced effect on ERK2 activity is consistent with the current understanding of ERK1 and ERK2 function.

While this study demonstrates that GP expression reduces ERK2 phosphorylation and activation, the data also provide evidence that increasing or decreasing active ERK2 levels inversely affects GP-induced rounding and detachment. While the events that ensue to induce cell death are unknown, ERK2 is a pivotal protein within the ERK/MAPK signaling cascade, and it is likely that downstream substrates of ERK2 mediate GP cytotoxicity. Once activated, the ubiquitously expressed ERK1 and ERK2 MAPKs modify a diverse array of substrates that relay signals mediating critical cellular responses (reviewed in reference 48). They phosphorylate approximately 160 proteins with substantial regulatory functions, including other protein kinases, transcription factors, cytoskeletal proteins, and other enzymes (10, 48). Reduced ERK2 activity during GP expression may lead to altered activity of one of these diverse substrates, thereby increasing cell rounding and detachment. The results from experiments utilizing constitutively active ERK2 indicate that at least one important substrate most likely localizes to the nucleus. The expression of a nuclear form of constitutively activated ERK2 decreased cell rounding and detachment, an effect not observed with a cytoplasmic version of active ERK2. A study by Robinson et al. demonstrated that certain functions of ERK2 are dependent on the nuclear localization of the active enzyme (32). In addition, it has been shown that adhesion to the extracellular matrix is required for efficient accumulation of activated ERK in the nucleus (4, 11). This finding suggests that the loss of adherence in GP-expressing cells decreases active and nuclear ERK2 levels. Substrates of nuclear ERK2 include several transcription factors which regulate the expression of a large array of genes involved in diverse cellular processes and could affect cell viability. At the same time, we cannot exclude the possibility that a cytoplasmic protein, such as a cytoskeletal substrate, may be the target of phosphorylation. Indeed, one possible cytoplasmic target of ERK2 is dynamin, an important

mediator of membrane internalization and integrin receptor endocytosis (12, 14, 21). Previous results have shown that a dominant negative version of this protein reduces GP-induced integrin downmodulation (41). However, we were unable to demonstrate an interaction between these two proteins (data not shown), suggesting that the observed effects of ERK activity on GP cytotoxicity are not mediated by dynamin.

Several viruses have been shown to modulate the ERK/MAPK signaling cascade, including influenza virus (31), borna disease virus (30), coxsackievirus (26), visna virus (6), human immunodeficiency virus (HIV) (28), vaccinia virus (13), Epstein-Barr virus (17), cytomegalovirus (33), and human herpesvirus 8 (1). These viruses stimulate the activation of this pathway, which results in efficient cell cycle promotion as well as high cellular and viral gene production. Fewer studies have demonstrated a negative correlation between ERK activity and viral protein expression such as we have described. One such study demonstrated that the hepatitis C virus nonstructural NS5A protein inhibits EGF-stimulated activation of the ERK pathway by inhibiting EGFR complex formation (27). A recent study by Yoshizuka et al. reported that the expression of the HIV type 1 (HIV-1) Vpr protein is associated with the downregulation of genes in the ERK/MAPK pathway and with decreased phosphorylation of ERK1/2 (49). Vpr is associated with the induction of a G_2/M cell cycle arrest. That study suggests that an alternative mechanism of HIV-1 Vpr-induced cell cycle arrest is the Vpr-associated decrease in ERK1/2 activation, and the authors speculate that this function is important for HIV replication and pathogenesis. Currently, little is known about the effects of Ebola virus GP expression on cell cycle progression. The findings with HIV-1 Vpr, along with the known regulatory effects of adhesion and ERK activity on the activation of cyclin-dependent kinases, which regulate the cell cycle (5, 24, 34), raise this possibility.

The results of this study demonstrate that Ebola virus GP reduces the phosphorylation and activation of the signaling molecule ERK2 and that reduction of ERK2 activity further enhances GP-induced αV integrin downmodulation, a correlate of cytopathicity. The observation that both GP-mediated cytotoxicity and ERK2 dephosphorylation are dependent on the mucin-like domain of GP further suggests that these processes may be intimately related. This is the first evidence of a GP-induced perturbation on a specific cellular signaling cascade. Future analysis of the activity of this signaling cascade during live viral infection may indicate an important role for this pathway not only in GP-mediated cytotoxicity but also in Ebola virus pathogenesis as well. A more complete understanding of the mechanisms involved in this phenomenon may facilitate the development of therapies for Ebola virus infection, which are currently unavailable.

ACKNOWLEDGMENTS

We thank Ati Tislerics for assistance with manuscript preparation, Toni Garrison and Brenda Hartman for figure preparation, and members of the G. J. Nabel and N. J. Sullivan labs for helpful discussions and advice.

This research was supported in part by the Intramural Research Program of the NIH, Vaccine Research Center, NIAID. J.-F.F. was supported by a fellowship from the Damon Runyon Cancer Research Foundation and by NIH grant N01 HV28183.

REFERENCES

1. **Akula, S. M., P. W. Ford, A. G. Whitman, K. E. Hamden, J. G. Shelton, and J. A. McCubrey.** 2004. Raf promotes human herpesvirus-8 (HHV-8/KSHV) infection. Oncogene **23:**5227–5241.
2. **Alazard-Dany, N., V. Volchkova, O. Reynard, C. Carbonnelle, O. Dolnik, M. Ottmann, A. Khromykh, and V. E. Volchkov.** 2006. Ebola virus glycoprotein GP is not cytotoxic when expressed constitutively at a moderate level. J. Gen. Virol. **87:**1247–1257.
3. **Aplin, A. E., B. P. Hogan, J. Tomeu, and R. L. Juliano.** 2002. Cell adhesion differentially regulates the nucleocytoplasmic distribution of active MAP kinases. J. Cell Sci. **115:**2781–2790.
4. **Aplin, A. E., S. A. Stewart, R. K. Assoian, and R. L. Juliano.** 2001. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. **153:**273–282.
5. **Assoian, R. K., and M. A. Schwartz.** 2001. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. Curr. Opin. Genet. Dev. **11:**48–53.
6. **Barber, S. A., L. Bruett, B. R. Douglass, D. S. Herbst, M. C. Zink, and J. E. Clements.** 2002. Visna virus-induced activation of MAPK is required for virus replication and correlates with virus-induced neuropathology. J. Virol. **76:**817–828.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Chan, S. Y., M. C. Ma, and M. A. Goldsmith.** 2000. Differential induction of cellular detachment by envelope glycoproteins of Marburg and Ebola (Zaire) viruses. J. Gen. Virol. **81:**2155–2159.
9. **Chan, S. Y., R. F. Speck, M. C. Ma, and M. A. Goldsmith.** 2000. Distinct mechanisms of entry by envelope glycoproteins of Marburg and Ebola (Zaire) viruses. J. Virol. **74:**4933–4937.
10. **Cobb, M. H., J. E. Hepler, M. Cheng, and D. Robbins.** 1994. The mitogen-activated protein kinases, ERK1 and ERK2. Semin. Cancer Biol. **5:**261–268.
11. **Danilkovitch, A., S. Donley, A. Skeel, and E. J. Leonard.** 2000. Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells. Mol. Cell. Biol. **20:**2218–2227.
12. **del Pozo, M. A., N. Balasubramanian, N. B. Alderson, W. B. Kiosses, A. Grande-Garcia, R. G. Anderson, and M. A. Schwartz.** 2005. Phosphocaveolin-1 mediates integrin-regulated membrane domain internalization. Nat. Cell Biol. **7:**901–908.
13. **de Magalhaes, J. C., A. A. Andrade, P. N. Silva, L. P. Sousa, C. Ropert, P. C. Ferreira, E. G. Kroon, R. T. Gazzinelli, and C. A. Bonjardim.** 2001. A mitogenic signal triggered at an early stage of vaccinia virus infection: implication of MEK/ERK and protein kinase A in virus multiplication. J. Biol. Chem. **276:**38353–38360.
14. **Echarri, A., and M. A. del Pozo.** 2006. Caveolae internalization regulates integrin-dependent signaling pathways. Cell Cycle **5:**2179–2182.
15. **Eliceiri, B. P., R. Klemke, S. Stromblad, and D. A. Cheresh.** 1998. Integrin alphavbeta3 requirement for sustained mitogen-activated protein kinase activity during angiogenesis. J. Cell Biol. **140:**1255–1263.
16. **Fischer, A. M., C. D. Katayama, G. Pages, J. Pouyssegur, and S. M. Hedrick.** 2005. The role of erk1 and erk2 in multiple stages of T cell development. Immunity **23:**431–443.
17. **Gao, X., K. Ikuta, M. Tajima, and T. Sairenji.** 2001. 12-O-Tetradecanoylphorbol-13-acetate induces Epstein-Barr virus reactivation via NF-kappaB and AP-1 as regulated by protein kinase C and mitogen-activated protein kinase. Virology **286:**91–99.
18. **Geisbert, T. W., H. A. Young, P. B. Jahrling, K. J. Davis, E. Kagan, and L. E. Hensley.** 2003. Mechanisms underlying coagulation abnormalities in Ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. J. Infect. Dis. **188:**1618–1629.
19. **Giancotti, F. G., and E. Ruoslahti.** 1999. Integrin signaling. Science **285:**1028–1032.
20. **Hatano, N., Y. Mori, M. Oh-hora, A. Kosugi, T. Fujikawa, N. Nakai, H. Niwa, J. Miyazaki, T. Hamaoka, and M. Ogata.** 2003. Essential role for ERK2 mitogen-activated protein kinase in placental development. Genes Cells **8:**847–856.
21. **Hinshaw, J. E.** 2006. Filling the GAP for dynamin. Nat. Cell Biol. **8:**432–433.
22. **Howe, A. K., A. E. Aplin, and R. L. Juliano.** 2002. Anchorage-dependent ERK signaling—mechanisms and consequences. Curr. Opin. Genet. Dev. **12:**30–35.
23. **Kiley, M. P., E. T. Bowen, G. A. Eddy, M. Isaacson, K. M. Johnson, J. B. McCormick, F. A. Murphy, S. R. Pattyn, D. Peters, O. W. Prozesky, R. L. Regnery, D. I. Simpson, W. Slenczka, P. Sureau, G. G. van der, P. A. Webb, and H. Wulff.** 1982. Filoviridae: a taxonomic home for Marburg and Ebola viruses? Intervirology **18:**24–32.
24. **Le Gall, M., D. Grall, J. C. Chambard, J. Pouyssegur, and E. Obberghen-Schilling.** 1998. An anchorage-dependent signal distinct from p42/44 MAP kinase activation is required for cell cycle progression. Oncogene **17:**1271–1277.
25. **Li, Y. Q., C. S. Hii, C. J. Der, and A. Ferrante.** 1999. Direct evidence that

ERK regulates the production/secretion of interleukin-2 in PHA/PMA-stimulated T lymphocytes. Immunity **96:**524–528.
26. **Luo, H., B. Yanagawa, J. Zhang, Z. Luo, M. Zhang, M. Esfandiarei, C. Carthy, J. E. Wilson, D. Yang, and B. M. McManus.** 2002. Coxsackievirus B3 replication is reduced by inhibition of the extracellular signal-regulated kinase signaling pathway. J. Virol. **76:**3365–3373.
27. **Macdonald, A., J. K. Chan, and M. Harris.** 2005. Perturbation of epidermal growth factor receptor complex formation and Ras signalling in cells harbouring the hepatitis C virus subgenomic replicon. J. Gen. Virol. **86:**1027–1033.
28. **Montes, M., N. E. Tagieva, N. Heveker, C. Nahmias, F. Baleux, and A. Trautmann.** 2000. SDF-1-induced activation of ERK enhances HIV-1 expression. Eur. Cytokine Netw. **11:**470–477.
29. **Pages, G., S. Guerin, D. Grall, F. Bonino, A. Smith, F. Anjuere, P. Auberger, and J. Pouyssegur.** 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science **286:**1374–1377.
30. **Planz, O., S. Pleschka, and S. Ludwig.** 2001. MEK-specific inhibitor U0126 blocks spread of Borna disease virus in cultured cells. J. Virol. **75:**4871–4877.
31. **Pleschka, S., T. Wolff, C. Ehrhardt, G. Hobom, O. Planz, U. R. Rapp, and S. Ludwig.** 2001. Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. Nat. Cell Biol. **3:**301–305.
32. **Robinson, M. J., S. A. Stippec, E. Goldsmith, M. A. White, and M. H. Cobb.** 1998. A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. Curr. Biol. **8:**1141–1150.
33. **Rodems, S. M., and D. H. Spector.** 1998. Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. J. Virol. **72:**9173–9180.
34. **Roovers, K., G. Davey, X. Zhu, M. E. Bottazzi, and R. K. Assoian.** 1999. Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. Mol. Biol. Cell **10:**3197–3204.
35. **Ryabchikova, E. I., L. V. Kolesnikova, and S. V. Netesov.** 1999. Animal pathology of filoviral infections. Curr. Top. Microbiol. Immunol. **235:**145–173.
36. **Saba-El-Leil, M. K., F. D. Vella, B. Vernay, L. Voisin, L. Chen, N. Labrecque, S. L. Ang, and S. Meloche.** 2003. An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. EMBO Rep. **4:**964–968.
37. **Sanchez, A., A. S. Khan, S. R. Zaki, G. J. Nabel, T. G. Ksiazek, and C. J. Peters.** 2001. *Filoviridae*: Marburg and Ebola viruses, p. 1279–1304. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott, Williams & Wilkins, Philadelphia, PA.
38. **Schwartz, M. A., and V. Baron.** 1999. Interactions between mitogenic stimuli, or, a thousand and one connections. Curr. Opin. Cell Biol. **11:**197–202.
39. **Schwartz, M. A., and M. H. Ginsberg.** 2002. Networks and crosstalk: integrin signalling spreads. Nat. Cell Biol. **4:**E65–E68.
40. **Simmons, G., R. J. Wool-Lewis, F. Baribaud, R. C. Netter, and P. Bates.** 2002. Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. J. Virol. **76:**2518–2528.
41. **Sullivan, N. J., M. Peterson, Z. Y. Yang, W. P. Kong, H. Duckers, E. Nabel, and G. J. Nabel.** 2005. Ebola virus glycoprotein toxicity is mediated by a dynamin-dependent protein-trafficking pathway. J. Virol. **79:**547–553.
42. **Takada, A., S. Watanabe, H. Ito, K. Okazaki, H. Kida, and Y. Kawaoka.** 2000. Downregulation of b1 integrins by Ebola virus glycoprotein: implication for virus entry. Virology **278:**20–26.
43. **Vantaggiato, C., I. Formentini, A. Bondanza, C. Bonini, L. Naldini, and R. Brambilla.** 2006. ERK1 and ERK2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J. Biol. **5:**14.
44. **Volchkov, V. E., H. Feldmann, V. A. Volchkova, and H.-D. Klenk.** 1998. Processing of the Ebola virus glycoprotein by the proprotein convertase furin. Proc. Natl. Acad. Sci. USA **95:**5762–5767.
45. **Wool-Lewis, R. J., and P. Bates.** 1998. Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. J. Virol. **72:**3155–3160.
46. **Yang, Z.-Y., R. Delgado, L. Xu, R. F. Todd, E. G. Nabel, A. Sanchez, and G. J. Nabel.** 1998. Distinct cellular interactions of secreted and transmembrane Ebola virus glycoproteins. Science **279:**1034–1037.
47. **Yang, Z.-Y., H. J. Duckers, N. J. Sullivan, A. Sanchez, E. G. Nabel, and G. J. Nabel.** 2000. Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. Nat. Med. **6:**886–889.
48. **Yoon, S., and R. Seger.** 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24:**21–44.
49. **Yoshizuka, N., Y. Yoshizuka-Chadani, V. Krishnan, and S. L. Zeichner.** 2005. Human immunodeficiency virus type 1 Vpr-dependent cell cycle arrest through a mitogen-activated protein kinase signal transduction pathway. J. Virol. **79:**11366–11381.